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Putting the brakes on the cell cycle: mechanisms of cellular growth arrest

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Abstract

Precise regulation of cellular proliferation is critical to tissue homeostasis and development, but misregulation leads to diseases of excess proliferation or cell loss. To achieve precise control, cells utilize distinct mechanisms of growth arrest such as quiescence and senescence. The decision to enter these growth arrested states or proliferate is mediated by the core cell-cycle machinery that responds to diverse external and internal signals. Recent advances have revealed the molecular underpinnings of these cell-cycle decisions, highlighting the unique nature of cell-cycle entry from quiescence, identifying endogenous DNA damage as a quiescence-inducing signal, and establishing how persistent arrest is achieved in senescence.

Introduction

One of the most fundamental decisions that a cell continuously makes is whether to proliferate or exist in a nondividing state. Cells integrate a wide variety of internal and external stimuli to determine if the cell-cycle machinery will be initiated. In multicellular organisms, defects in the transition between proliferation and arrest lead to diseases of excess proliferation (cancer) or cell loss (aging and degeneration). To address the complex organismal needs for proper tissue development and homeostasis, there exists great diversity in the forms and functions of growth arrest. Recent work has improved our understanding on how cells integrate signaling information to regulate cell-cycle arrest.

Broadly, growth arrest is categorized as either quiescence, terminal differentiation, or senescence. Quiescence is a non-proliferative state induced by a variety of stimuli including mitogen starvation, contact inhibition, and loss of adhesion. A defining characteristic of quiescence is the ability of cells to re-enter the cell cycle following the reversal of these signals. Quiescent cells are resistant to stress and toxicity, a trait that is critical to the maintenance of tissue stem cells. While some aspects of the quiescence program are shared,

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Conflict of Interest

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there are unique properties that distinguish the response to different quiescence-inducing signals. Contrary to the reversible nature of cell-cycle exit in quiescence, terminal differentiation entails permanent exit from the cell cycle. Classically, cellular senescence entails permanent exit combined with persistent stress (e.g., oncogene activation, telomere shortening). Initially thought to function as an important safeguard against uncontrolled cellular proliferation and cancer, cellular senescence now has many appreciated roles in development (e.g., renal morphogenesis), tissue homeostasis (e.g., wound healing), and aging [1].

In this review, we focus on three regulatory aspects of quiescence and senescence as they relate to mammalian cells. Terminal differentiation has many similarities to quiescence but with more permanent repressive mechanisms that are not discussed here. First, we discuss how ongoing studies have clarified the molecular mechanisms and systems properties underlying the classical concept of the restriction point in quiescence. Next, we consider how cells utilize transient quiescence to tolerate the inherently error prone nature of replicating the genome. Finally, we compare the quiescence and senescence growth arrest programs, evaluating the mechanisms that promote and maintain the irreversible arrest characteristic of senescence. For each section, we summarize the relevant arrest-inducing stimuli and describe the core machinery functioning in induction of arrest, maintenance of arrest, and re-entry into the cell cycle.

Quiescence Decisions and the Restriction Point

The decision to enter quiescence versus proliferate relies on the funneling of diverse and complex signals into a core cell-cycle machinery [2,3]. Critical to cell-cycle entry is the phosphorylation of the pocket protein family – including p107, p130, and retinoblastoma protein (Rb) – of which we will focus on the regulation of Rb in this review. When unphosphorylated, Rb binds to chromatin and prevents E2F transcription factors from inducing genes essential for proliferation (Figure 1). Under pro-growth conditions, Cyclin-dependent kinases 4 and 6 (CDK4/6) are activated by the binding of cyclin D (D1, D2, D3) and phosphorylate Rb, releasing it from chromatin and enabling E2F target transcription. Activation of Cyclin-dependent kinase 2 (CDK2) following binding by E2F-target cyclin E (E1, E2) helps to reinforce and maintain Rb phosphorylation and cell-cycle progression. The pro-proliferative effects of CDK4/6-cyclin D and CDK2-cyclin E complexes are opposed by anti-proliferative proteins. The CDK interacting protein/kinase inhibitory protein (CIP/KIP) family of inhibitors, including p21, p27, and p57, inhibit both CDK4/6 and CDK2 complexes, while the inhibitor of kinase 4 (INK4) family of inhibitors, including p16, specifically inhibit CDK4/6. Discovery of how this core molecular machinery contributes to cell-cycle regulation is the foundation for recent work investigating the different forms of cellular growth arrest.

Cell-cycle exit occurs via the induction of CDK inhibitors and/or the downregulation of cyclins and CDKs. For example, serum starvation results in an upregulation of p27 and a suppression of the G1 cyclins, cyclin D and cyclin E. Thus, the quiescent state is defined by low CDK activity and hypo-phosphorylation of Rb protein. Despite their defined roles in cell-cycle entry, previous genetic knockout experiments in mice have shown that the

majority of cells proliferate normally in mouse embryos lacking all three cyclin D proteins (D1−/−D2−/−D3−/−) or lacking cyclin E1 and E2 (E1−/−E2−/−) [4]. These results led to the current model that both types of G1 cyclins can adequately promote Rb phosphorylation and that a single G1 cyclin is sufficient for mammalian cell proliferation. Recently, this model has been tested by genetic ablation of all D-type and E-type cyclins. While some cell types (e.g., mouse embryonic fibroblasts) failed to proliferate, other cell types, including embryonic stem cells, proliferated normally in the absence of all G1 cyclins, likely by utilizing cyclin A2 [5]. Nevertheless, the loss of G1 cyclins attenuated the pluripotent characteristics of the stem cells. This result is reminiscent of previous findings that prolonged overexpression of CDK inhibitors results in the differentiation of human embryonic stem cells [6]. However, the ablation of all G1 cyclins by Liu *et al* illuminates that pluripotency is not primarily affected by a lack of proliferation but rather by a lack of G1 CDK activity, which they show directly phosphorylates pluripotency factors Nanog, Oct4, and Sox2 to promote their stability. In contrast to direct inhibition of CDKs by CDK inhibitor overexpression, cell-cycle arrest by quiescence-inducing signals (contact inhibition, serum starvation) suppresses terminal differentiation [7]. This contrast indicates unique signaling mechanisms present in quiescence to promote the stability of the pluripotency factors or inhibit differentiation transcriptional programs, and may imply that the mechanism of quiescence induction could dictate the sensitivity of cells toward terminal differentiation.

Before cells can exit quiescence, there must be a reversal of the quiescence-inducing signals, for example by the reintroduction of growth factor signaling or lowering of cell density. In classic work, Pardee tested the requirement for mitogen signaling in cell-cycle entry from a serum-starved, quiescent state by restimulating with mitogens for various amounts of time. A point termed the “restriction point” was identified where cells will progress through to division even if growth factors are subsequently removed [8]. Later, use of E2F transcriptional reporters demonstrated that the restriction point approximately coincides with the activation of E2F [9]. More recently, the effects of prolonged arrest in quiescence have been investigated in both contact inhibited or serum starved quiescence cells: cells that were arrested for progressively longer time periods require stronger growth factor stimulation and more time to reach the restriction point [10]. Moreover, cellular re-entry into the cell cycle from quiescence is heterogeneous, whereby individual cells exhibit significantly different rates of restarting the cell cycle. The application of live-cell sensors, including the development of CDK2, E2F, and APC/C^{CDH1} activity reporters, have been critical to observing the heterogeneous nature of cellular re-entry [9–15]. One factor that may contribute to this heterogeneity is memory of previous cell growth conditions at quiescence induction [16]. Other inherent properties of quiescence may also contribute to this heterogeneity, and more work is required to understand how and why quiescence exit is so distinct from re-entry into the cell cycle for continuously cycling cells.

While cells coming out of quiescence have a G1 restriction point, recent work using single-cell imaging has challenged whether this model is true in continuously cycling cells. Following mitosis, cells either enter a reversible, CDK2-activity low, mitogen-sensitive state or immediately begin to increase CDK2 activity, entering G1 in a mitogen-insensitive state [12]. The cells born with high CDK2 activity have largely committed to the cell cycle when tested by mitogen removal. Thus, rather than a restriction point in G1, rapidly cycling cells

can commit to the cell cycle in the G2 phase of mother cells [17,18]. However, whether primary cells are subject to a G1 restriction point is still debated [17,18]. Although cells are no longer sensitive to mitogens past the restriction point, recent work has shown that acute cellular stress can still cause cell-cycle exit after the mitogen restriction point [15]. Treatment of cells with multiple stress-inducing conditions, including DNA damage, hyper-osmotic stress, and reactive oxygen stress, in combination with live cell imaging using the APC/C^{CDH1} activity reporter, identified that the molecular basis for the point of no return for stress sensitivity is the rapid, bi-stable inactivation of anaphase promoting complex (APC/C^{CDH1}) [15,19]. APC/C^{CDH1} inactivation is mediated by a dual negative feedback loop between APC/C^{CDH1} and Emi1, an E2F target gene [20].

There are clear differences in the regulation of G1 in cells coming out of quiescence compared to continuously cycling cells. The delayed and heterogeneous cell-cycle entry from quiescence may function to ensure resources for continued cellular division before leaving the protection of the quiescent state. It also may function to control the rate of tissue replenishment. Multiple mechanisms could account for this delay, including reversal of the global changes (transcription, metabolism, or epigenetics) that occur in quiescent cells [7,21–23]. The requirement for significant changes in the expression of the cell-cycle machinery, including decreases in CDK inhibitors and increases in cyclins as well as the CDKs themselves, may also contribute to this delay upon quiescence exit. Future work interrogating the differences between cycling cells and cell-cycle re-entry from quiescence will help determine how these regulatory mechanisms underlie cellular proliferation.

DNA Replication Stress-Mediated Quiescence

In addition to external signaling cues, quiescence can be initiated by internal cellular stress. One form of endogenous cellular stress is the intrinsically error prone nature of DNA replication. Historically, the DNA damage response has been well characterized for exogenous stress, in which DNA damage has been shown to mediate cell-cycle exit through the stabilization of p53 and the upregulation of its transcriptional targets, including p21 [24]. Recent work, outlined in this section, has shown that this same pathway senses and responds to endogenous DNA replication stress [25–28], which is caused by stalled replication forks that fail to restart or collapse [29–31]. It has been estimated that one to two forks stall every S phase in unperturbed cells [32,33]. Thus, endogenous DNA replication stress significantly impacts cellular proliferation.

In unperturbed cycling cells, cells either immediately increase CDK activity and enter the next cell cycle following mitosis, or exit the cell cycle into a quiescent state, defined by low CDK activity and significantly higher levels of p21. Quiescence entry depends on p21 expression, as p21−/− cells rarely enter the CDK low state [12,34]. Using single-cell imaging techniques coupled to tools like endogenously tagged p53 or p21 cell lines and fluorescently-tagged 53BP1, recent work has demonstrated that endogenous DNA replication stress generates this heterogeneity in p21 levels [26–28]. Higher levels of p53 and p21 are observed in cells that enter quiescence compared to cells that continue to cycle. These increased levels correlate with increased markers for DNA damage, including 53BP1 and γH2AX.

While endogenous DNA damage during S phase results in increased p53, it is not until G2 of mother cells and G1 of daughter cells that heterogeneity in p21 levels is observed. Both modeling and experimental testing of the timing of DNA damaging drugs revealed that the sensitivity of cells to DNA stress weakens as cells progress through G1 [35]. Critical for tolerating and repairing replication stress, E2F transcriptional activity regulates a variety of checkpoint and replication repair proteins [36]. If not repaired, memory of DNA damage incurred in S phase of the mother cell is passed on to daughter cells, either through DNA damage signaling molecule p53 [28] or through persistence of the damage itself [26,27].

Endogenous DNA replication stress doesn't solely dictate the proliferation versus quiescence decision. Rather, Rb phosphorylation is ultimately controlled by a stoichiometric competition between the induction of p21 by endogenous DNA damage stress and the induction of cyclin D controlled by mitogen signaling [28]. This stoichiometric competition converts two graded responses to DNA damage stress and mitogen signaling into an ultrasensitive activation of CDK4/6 [28,35]. Cells are maintained in quiescence as long as p21 levels exceed cyclin D1 levels. To activate CDK4/6 and exit quiescence, either the DNA damage is resolved, decreasing p21 levels, or mitogen signaling is strengthened, increasing cyclin D1 levels above p21 levels. While previous reports have identified positive roles for CDK inhibitors in the cell cycle (reviewed previously) [37], the recent stoichiometric competition observed implies that CDK4/6-cyclin D-p21 complexes are mostly inactive.

It has previously been reported that most cell types in p21-null, p27-null, and p21- p27- double knockout mice lack severe phenotypes [38]. Moreover, while p57 knock-out mice are inviable, mice in which p57 is replaced by p27 are viable and lack most developmental defects characteristic of p57-null mice [39]. These results contrast the model that any one CIP/KIP inhibitor is required in proliferation decisions. We suggest that cell-cycle entry decisions do not rely on a single cell-cycle inhibitor, but these decisions rely on the expression levels of all CDK inhibitors. An attractive model is that different pro- and anti-growth signals are integrated by their combined effects on the expression of CDK activators and inhibitors (Figure 2). We hypothesize that besides DNA damage, other extrinsic and intrinsic anti-growth signals could similarly induce transient quiescence. However, these signals may regulate cell-cycle entry through control of alternative cell-cycle regulators, not necessarily p21.

Mechanisms of cell-cycle arrest in senescence

The senescence phenotype is heterogeneous, with many described stimuli, changes to cellular physiology, and tissue/organismal effects that are beyond the scope of this review but are covered in detail in many excellent recent articles [1,40,41]. An extensive number of stimuli have been reported to trigger cellular senescence: oncogene activation [42,43], ionizing radiation and other DNA damaging agents, mitochondrial dysfunction [44,45], chromatin alterations [46–48], and impairment of ribosome biogenesis [49,50], for example. However, a fundamental trend does appear to link together these diverse stimuli: a potent cellular stressor induces persistent damage signaling, causing permanent cell-cycle arrest.

Activation of both the DNA damage response p53-p21 pathway and the Rb cell-cycle inhibitory pathway is a long-recognized theme of senescence. As in several forms of

quiescence, activation of the DNA damage response pathway is the initial stimulus triggering cell-cycle arrest. However, senescence appears to be unique from quiescence in the persistence of the DNA damage response: while the DNA damage response (DDR) is often transient in quiescence, recent evidence suggests that the DDR is persistently activated in senescence [51–53]. In both senescence and quiescence, upregulation of p21 enables robust cell-cycle exit via CDK4/6 inhibition. Unique to senescence is an eventual decline in p21 levels several days following the senescence-inducing stimulus combined with a concomitant increase in p16 protein levels, presumably to maintain CDK4/6 inactivation [46,54,55]. Extremely high affinity binding of p16 to CDK4/6 ensures suppression of CDK4/6 activity and contributes to the irreversible nature of cell-cycle arrest in senescence (Figure 3) [56,57]. Interestingly, in contrast to quiescence, G1 cyclin levels frequently remain high in senescent cells, though the significance of this is unknown.

Recently, numerous other changes to cellular physiology have been discovered. Changes to cellular chromatin architecture help to reinforce the senescent state. Senescence-associated heterochromatic foci transcriptionally silence E2F target genes, decreasing cellular sensitivity to mitogenic stimuli [58,59]. Recent work employing Hi-C to investigate genome architecture demonstrated that senescent cells undergo broad changes in global chromatin organization [60,61]. This change appears to be due in part to histone and nuclear lamin depletion [62–64], as well as decreased tethering of chromatin to the nuclear lamin [65,66]. Alterations in chromatin dynamics may contribute to increased transcription of inflammatory cytokines in senescent cells [67,68]. Several recent studies employing both cellular and mouse models of radiation- or oncogene-induced senescence demonstrated that cytoplasmic chromatin fragments accumulate in senescent cells, activating the pro-inflammatory cGAS-STING pathway and contributing to a pro-inflammatory phenotype [69–71]. Secretion of inflammatory proteins, termed the senescence associated secretory phenotype (SASP), functions as a pro-senescence stimulus in both an autocrine and paracrine manner [1]. cGAS activation and the SASP phenotype appear to be unique to senescence and not found in quiescent cells. Moreover, while quiescent cells reside in a state of low metabolic activity, senescent cells remain metabolically active. However, metabolic profiling and functional studies of senescent cells indicate that senescent cell metabolism differs significantly from proliferating cells [45,72,73]. These multitude of changes to cellular physiology appear to be important in reinforcing the growtharrest of senescent cells.

Although senescence is generally considered to be an irreversible cell-cycle arrest, several studies have suggested mechanisms to alter the fate of senescent cells. It was previously known that acute genetic deletion of Rb and Rb-related proteins induces cell-cycle re-entry in fibroblasts [74]. Inactivation of p53 was also shown to promote senescence escape, highlighting the role for a persistent DNA damage response in senescence maintenance [75,76]. More recent work has demonstrated that senescent cells exist on the verge of apoptosis, spurring efforts to pharmacologically eliminate senescent cells by triggering the apoptotic pathway [54,77,78]. Finally, genetically reprogramming senescent cells through an iPSC state has recently been demonstrated to ameliorate features of senescence and aging both *in vitro* and *in vivo*, suggesting that increased stemness may be a route out of the senescent cell state [79,80]. Future work identifying mechanisms of cellular rejuvenation

and cell-cycle re-entry will provide greater insight into the mechanisms of senescence maintenance and may ultimately have therapeutic potential.

Conclusions

The similarities between quiescence and the first several days following a senescence-inducing stimulus suggest that there may be a critical cellular decision window during which a cell can either enter a temporary quiescent state or initiate permanent cell-cycle exit via a senescence program. Moreover, important factors have been identified that are critical in protecting quiescent cells from senescence and differentiation [81]. Future work will focus on identifying the core decision-making machinery and the signals that a cell integrates when making this decision. Moreover, to date, the majority of studies of cellular senescence have examined phenotypes at the population level. Future studies examining cell-to-cell heterogeneity within a senescent population will help to unravel the complexities underlying senescence.

While the stimuli and mechanisms of cell-cycle arrest are diverse, common principles have emerged. Essential to any form of arrest is inhibition of cyclin-dependent-kinase activity, especially the G1 kinases CDK4 and CDK6, which play a critical role in promoting cell-cycle entry. Control over CDK activity therefore appears to be the ultimate determinant of cell-cycle arrest. Future work uncovering additional mechanisms of CDK regulation, and how these mechanisms dictate which form of cell-cycle arrest cells enter, will continue to advance the field.

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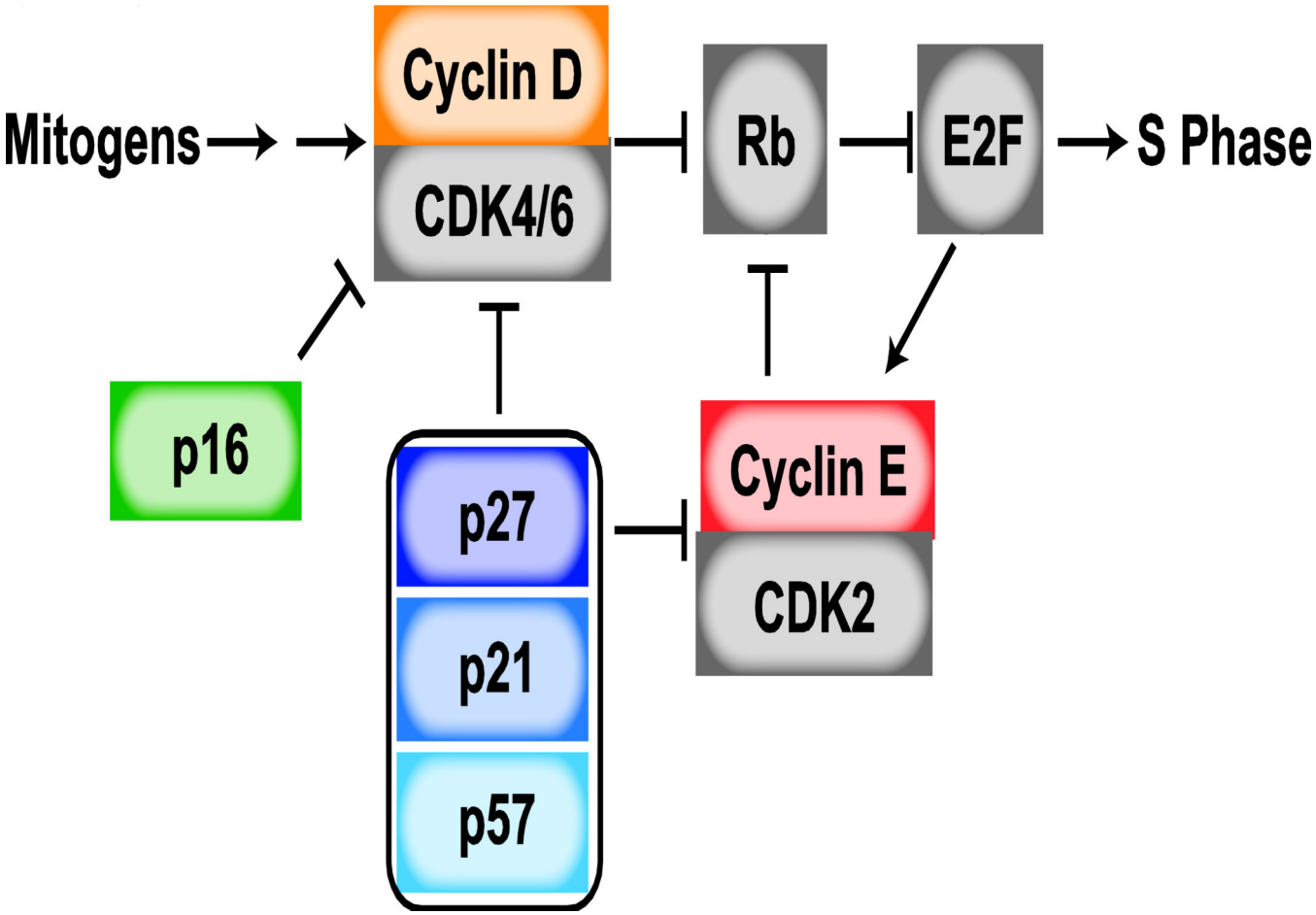

Figure 1: Cell-Cycle Regulation in G1 phase.

Progression through the cell cycle depends upon the activation of cyclin-dependent kinases (CDK), whose activity requires the binding of specific regulatory subunits, known as cyclins. CDK4/6 is activated by the binding of D-type cyclins, whose expression are controlled by mitogenic growth factors. CDK2 is activated by E-type cyclins, known transcriptional targets of E2F. The activity of both CDK4/6 and CDK2 complexes are opposed by CIP/KIP CDK inhibitors, including p21, p27, and p57, while INK4 inhibitors like p16 exclusively bind CDK4/6, preventing cyclin D-CDK4/6 interaction. CDK activity promotes cell-cycle entry through the phosphorylation and inactivation of the transcriptional repressor, Rb. Following phosphorylation of Rb, E2F transcription is activated, promoting cell-cycle progression.

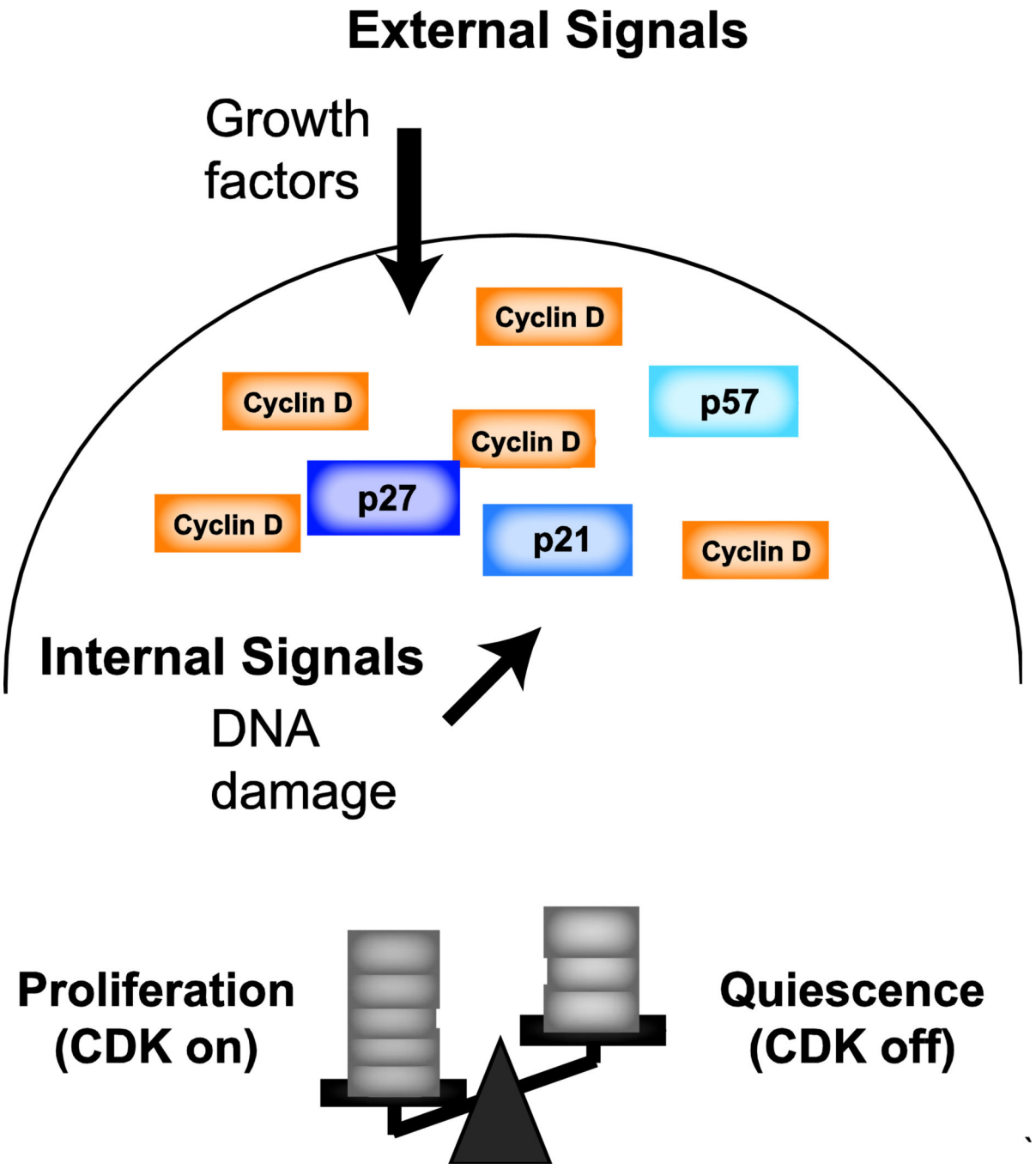

Figure 2: Model for Integration of Cell-Cycle Inputs.

External and internal signals regulate the core cell-cycle machinery, including cyclins and CDK inhibitors. While mitogenic signals are known to regulate cyclin D expression, recent work has shown that endogenous DNA damage leads to the upregulation of p21. Additionally, extrinsic and intrinsic growth signals contribute to cell-cycle decisions. We propose a model in which the cell cycle decisions rely on the combined effects of pro-and anti-growth signals and their integration by their combined effects on the expression of

CDKs, cyclins, and CDK inhibitors. This model is consistent with an ultrasensitive activation of CDKs.

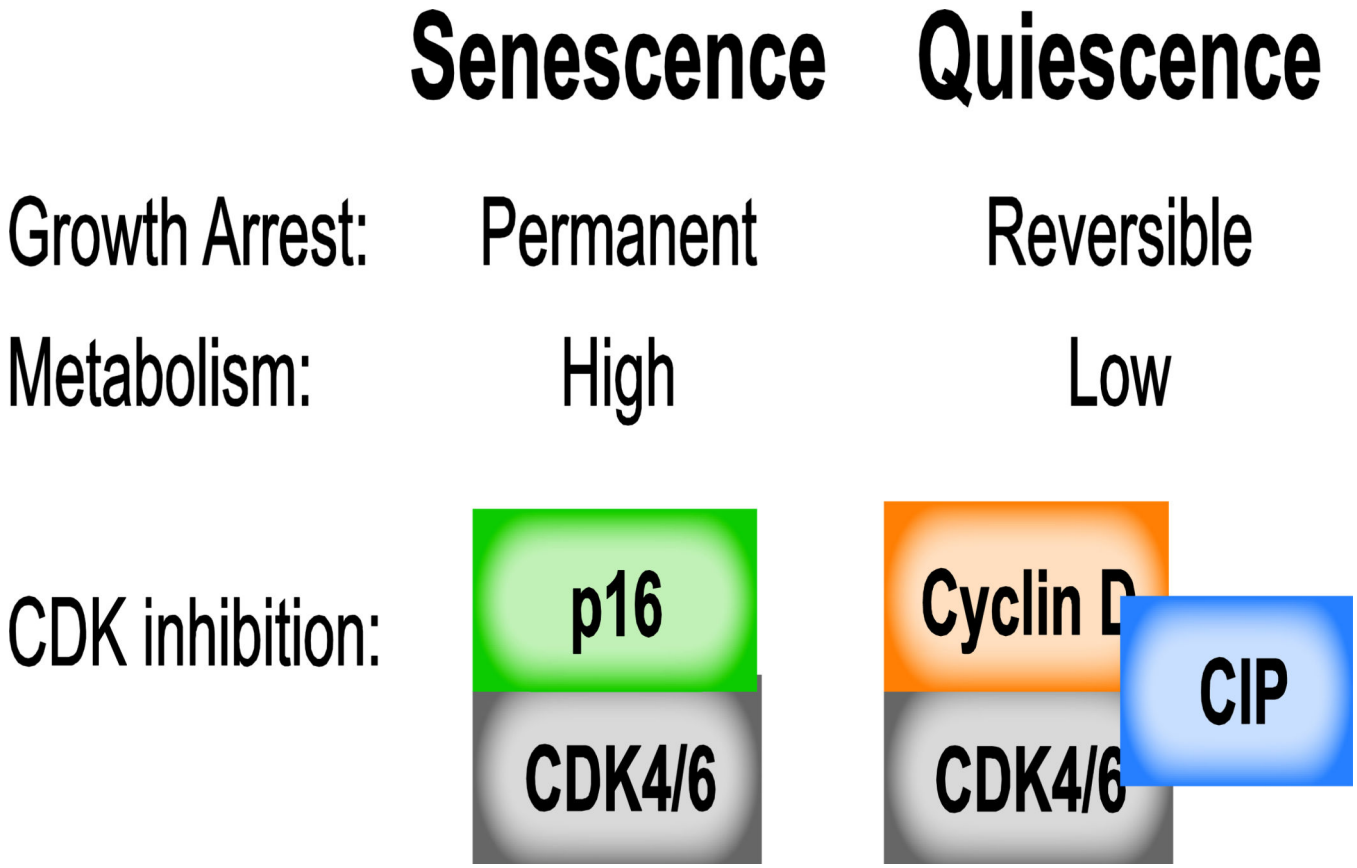

Figure 3: Comparing Senescence and Quiescence.

Contrary to the reversible nature of quiescence, senescence is a permanent growth arrest. Fundamental differences between these two types of growth arrest include continued high metabolism in senescent cells compared to the low metabolic state of quiescent cells. Moreover, senescent cells rely on p16 to inhibit CDK4/6, while quiescent cells utilize the CIP/KIP inhibitors to bind and inhibit CDK cyclin complexes.